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Original Article

Chebulagic acid reduces inflammation and regulates enteric glial cells in mouse models of dextran sulfate sodium-induced colitis

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Abstract: Objective: The aim of this study was to investigate whether Chebulagic acid (CA) can improve inflammation-induced glial dysregulation. Methods: CA was administered intraperitoneally in dextran sulfate sodium (DSS)-induced colitis. Disease activity index and histological scores were evaluated. Expression of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), glial-derived neurotrophic factor (GDNF), p38 mitogen-activated protein kinase (p38 MAPK), and myeloperoxidase activity were tested by ELISA assay. Expression of glial fibrillary acidic protein (GFAP) and p38 MAPK was analyzed by Western blot assay and immunohistochemical staining. Results: Defective glial function following inflammation was observed. CA remarkedly ameliorated experimental colitis and significantly increased expression of GFAP and GDNF, in part, through downregulation of TNF-α, IL-1β and phosphorylated p38 MAPK expression and reduction of infiltration of leukocytes. Conclusion: CA activates enteric glia and reduces inflammation in DSS-induced colitis. CA may be a promising therapeutic agent to selectively target enteric glial cells in patients with ulcerative colitis.

Keywords: Chebulagic acid, inflammation, enteric glial cell, ulcerative colitis, dextran sulfate sodium-induced colitis

Introduction

Enteric glial cells (EGCs) are critical regulators of gut homeostatic functions in ulcerative colitis (UC), a chronic idiopathic inflammatory disease that primarily affects the colon [1, 2]. Alterations of EGCs may participate in the initiation and perpetuation of UC. Previous studies have shown that EGCs are altered in UC [3, 4]. Therefore, selective enteroglial-specific targeting in UC is of clinical significance.

It has been established that EGCs are altered in patients with UC, but mechanisms responsible for gliosis remain controversial, though likely involving inflammation [3, 4]. In fact, a series of studies from human and animal models have consistently shown a causal relationship between the presence of gastrointestinal inflammation and EGCs proliferation [5, 6]. Accordingly, effective inhibition of gut inflammatory response might be of therapeutic potential for structural and functional abnormalities of the glial system associated with inflammation.

Chebulagic acid (CA), one of the main bio-active constituents of *Terminalia chebula* and *Phyllanthus emblica*, is a benzopyran tannin compound with various kinds of medicinal potential, including anti-oxidative, anti-inflammatory, and neuroprotective activities [7-9]. In Traditional Chinese Medicine, the fruit of *Terminalia chebula* has commonly been used for treating diarrheal diseases. Therefore, it is speculated that CA may be a potential therapeutic approach in treatment for glial abnormality of patients with UC. Unfortunately, to date, there has been no information on whether CA is therapeutic for gut glial abnormalities in patients with UC. Therefore, this study hypothesized that CA could improve gut glial abnormalities through its anti-inflammatory effects and neuronal pro-

Table 1. Effects of CA on clinical indices and histological injury scores

Group	Number	DAI	Histological scores
Normal control	8	0	0
DSS	8	1.04±0.97*	7.88±0.93*
Low-dose CA	8	0.54±0.16	5.88±0.60
High-dose CA	8	0.50±0.17	5.53±0.78

Results are expressed as $\bar{x}$±s. *P<0.05 (versus normal control group and CA group).

tective actions. This study investigated whether inflammatory response is responsible for impairment of EGCs and whether CA could regulate colonic glial abnormalities in a murine model of dextran sulfate sodium (DSS)-induced colitis, which resembles human UC.

Materials and methods

Animals

Six to eight-week-old male BALB/c mice, weighing about 18-20 g, were purchased from Tengxin Biotechnology Co., Ltd., Chongqing, PR China. They were housed in cages at room temperature (23°C), with humidity of 40% and alternating 12:12-hour light-dark cycles. Standard mouse chow pellets and drinking water were supplied ad libitum. This experiment was approved by the Animal Ethics Committee of Guizhou Provincial People's Hospital, Guizhou Medical University.

Induction of colitis and administration of CA

Experimental colitis was induced by administrating 5% DSS (molecular weight 36,000-50,000 Da, MP Biomedicals, Inc., Aurora, OH, USA) orally in drinking water for 7 days, followed by 1 day of normal drinking water. Seven days after induction of colitis, mice were randomly divided into 4 groups with 8 mice per group. In the first group, designated as the normal control group, colitis was not induced. No therapeutic intervention was administered. The second group, designated as the DSS group, received 5% DSS and saline solution. The third group, defined as the low-dose CA group, received 5% DSS and low-dose CA (10 mg/kg, Zhenye Biotechnology Co., Ltd., Shanghai, PR China). The fourth group, serving as the high-dose CA group, received 5% DSS and high-dose CA (20 mg/kg). In the third and fourth groups, mice were injected intraperitoneally with CA [10 or 20 mg/kg dissolved in phosphate buffered saline (PBS), 0.3 mL]. Doses were chosen based on previous studies and observations in preliminary experiments [10]. Mice were killed under anesthesia 12 days after CA therapy.

Evaluation of colitis

Disease activity index (DAI) was calculated by scoring changes in weight loss, stool occult blood, gross bleeding, and stool consistency, in accordance with methods reported by Murthy et al. [11]. After the mice were killed under anesthesia, colons were immediately isolated and fixed in 10% buffered formalin, paraffin-embedded, sectioned, and stained with hematoxylin and eosin (H&E). Histological scores of H&E-stained sections were assessed by two pathologists, in a blinded fashion, according to methods described by ten Hove et al. [12]. Mean scores in each section were calculated.

Myeloperoxidase (MPO) activity

MPO activity, an index of leukocyte recruitment, was determined with a MPO assay kit, according to manufacturer instructions (CytoStore, Alberta, Canada).

Enzyme-linked immunosorbent assay (ELISA)

Levels of glial-derived neurotrophic factor (GDNF), phosphorylated p38 mitogen-activated protein kinase (p-p38 MAPK), TNF-α, and IL-1β in homogenized colonic tissue were measured by ELISA, according to manufacturer instructions (Elabscience, Co., Ltd., Wuhan, PR China).

Immunohistochemistry

Colonic specimens were fixed in 10% formalin and embedded in paraffin. Sections (3 μm thick) were deparaffinized and rehydrated in degraded concentrations of ethanol. Endogenous peroxidase activity was blocked with 1.5% hydrogen peroxide in methanol for 30 minutes at room temperature. Nonspecific binding was blocked with 1% bovine serum albumin. Incubation with the glial fibrillary acidic protein (GFAP) antibody (1:500, Abcam, UK, specific markers of EGCs) or p38 MAPK antibody (1:200, Abcam, UK) was performed overnight at 4°C. The following day, sections were washed three times in PBS and incubated with

Table 2. Effects of CA on MPO activity, IL-1β, and TNF-α protein expression

Group	Number	MPO activity (units/mg)	IL-1β (pg/ml)	TNF-α (pg/ml)
Normal control	8	2.63±0.24	30.54±6.47	48.39±4.42
DSS	8	56.48±7.65*	163.78±32.61*	195.06±26.94*
Low-dose CA	8	17.93±5.20	79.83±10.22	98.90±9.07
High-dose CA	8	16.47±4.46	52.31±8.57**	75.04±10.89**

Results are expressed as $\bar{x}$±s. *P<0.05 versus Normal control and CA group; **P<0.05 versus Low-dose CA group.

Table 3. GDNF expression in colonic tissues

Group	Number	GDNF (ng/ml)
Normal control	8	19.48±5.17
DSS	8	8.02±2.50*
Low-dose CA	8	11.30±3.09
High-dose CA	8	26.29±9.04

Results are expressed as $\bar{x}$±s. *P<0.05 versus Normal control and high-dose CA group.

the biotinylated secondary antibody (1:200, Dako, USA). Finally, after washing with PBS again, the sections were stained in diaminobenzidine tetrahydrochloride (Sigma, St Louis, USA) with 0.03% hydrogen peroxide until a brown reaction product could be observed. Negative controls received the same treatment, except for the primary antibody. Positive cells were counted by analyzing 100 brown-stained cells in five separate fields randomly selected at magnification (×400).

Western blot analysis

To examine GFAP expression in colonic tissue, extracts from colonic tissue were prepared as, reported previously [13]. They were analyzed by Western blot, according to the standard method [14], by using anti-GFAP (1:10000) and anti-β-actin antibodies (1:150), respectively. All antibodies were purchased from ABCAM Biotechnology (Abcam, UK). Immune complexes were detected with the ECL system (Amersham Pharmacia Biotech Inc, Arlington, USA) and bands were measured by a FluorChem imaging system for quantitative densitometric analysis (Alpha Innotech Corp, San Leandro, CA, USA).

Statistical analysis

Data are expressed as $\bar{x}$±s and were analyzed by Student's t-test or analysis of variance (ANOVA). Differences are considered statistically significant when P values <0.05.

Results

Effects of CA on clinical indices and histological injury scores

Treatment with CA significantly ameliorated experimental colitis in mice, as assessed by DAI and histological injury scores. As shown in **Table 1**, compared with the control group, DAI scores were increased markedly in mice from the DSS group (P<0.05). In contrast, treatment with low or high doses of CA significantly decreased DAI scores in mice with DSS-induced colitis. Consistent with clinical features, administration of low or high doses of CA led to marked improvement in histological injury scores in DSS-treated mice, compared to the DSS group (P<0.05). However, there were no statistical differences between the low-dose CA group and high-dose group, regardless of DAI or histological injury scores.

Effects of CA on MPO activity

MPO activity was used to assess acute inflammatory infiltrate in the colonic tissue. As shown in **Table 2**, MPO activity levels were low in colonic tissues of normal control mice but markedly higher in mice with DSS-induced colitis. Greater MPO activity in mice with DSS-induced colitis was significantly reduced after administration of CA. Nevertheless, no statistical differences were observed between the low-dose CA group and high-dose group.

Effects of CA on pro-inflammatory cytokine expression

As shown in the **Table 2**, compared to the control group, a significant increase in pro-inflammatory cytokine expression of IL-1β and TNF-α, as assessed by ELISA, was observed in mice with DSS-induced colitis (P<0.05). The increase in the amount of IL-1β and TNF-α protein in mice with DSS-induced colitis was reduced by treatment with CA (P<0.05). In addition, statistical differences were observed between the low-dose CA group and high-dose group (P< 0.05).

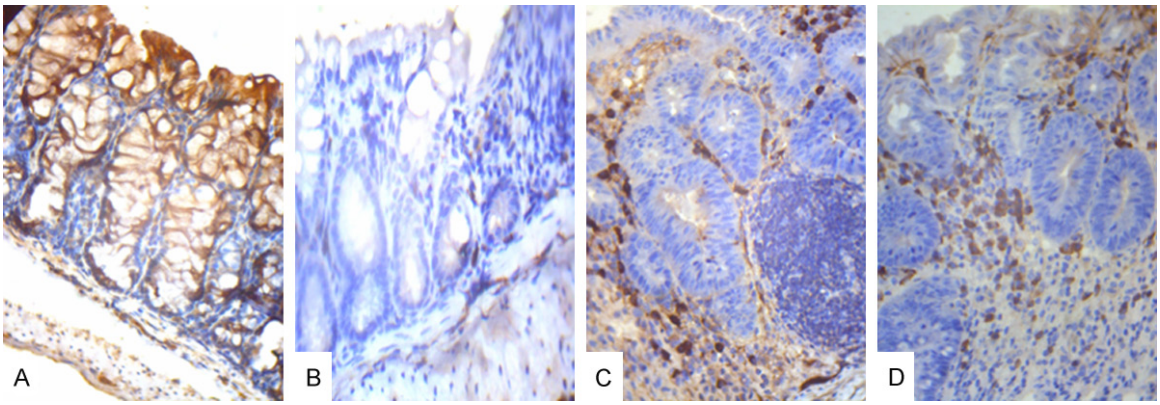

Figure 1. Effects of CA on GFAP-positive cells. Immunohistochemical staining of GFAP in colonic tissues (magnification ×400). A. Normal control group; B. DSS group; C. Low-dose CA group; D. High-dose CA group. Compared with that of normal controls and DSS group, colon of CA-treated mice showed a significant increase in the number of GFAP-positive cells (P<0.05). Most of the GFAP-positive cells displayed cytoplasmic staining.

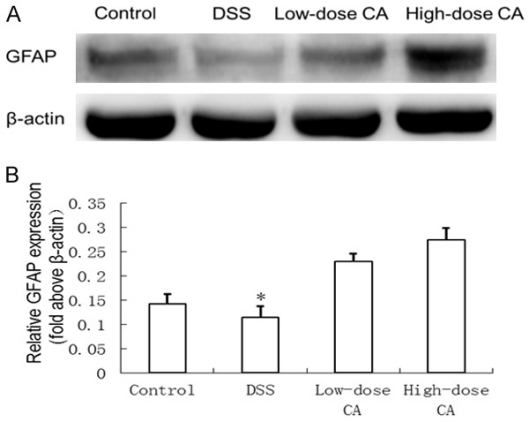

Figure 2. Effects of CA on GFAP protein expression. A. Western blot results display protein expression of GFAP in the colonic tissues. B. Densitometric analysis of expression of GFAP. DSS treatment decreased expression of GFAP, which was increased significantly with CA. Data are expressed as x±s (each group, n=8). *P<0.05 (versus control group, Low-dose CA group, and High-dose group).

Effects of CA on enteric glia

As shown in the **Table 3**, compared to the control group, a significant decrease in GDNF expression, as assessed by ELISA, was observed in mice with DSS-induced colitis (P<0.05). This decrease in the amount of GDNF in mice with DSS-induced colitis was increased by treatment with high-dose CA (P<0.05). However, treatment with low-dose CA had little effect on expression of GDNF in DSS-fed mice (P>0.05).

As shown in **Figure 1**, some GFAP-positive cells, stained tan, were detected in normal control mice, whereas DSS treatment decreased the number of GFAP-positive cells. In contrast, after treatment with CA, percentages of GFAP-positive cells increased significantly in mice with DSS-induced colitis, compared to the DSS group (P<0.05). In addition, this study also detected expression of GFAP, a specific marker of EGCs, using Western blot analysis. As shown in **Figure 2**, GFAP protein levels in mice from the DSS group were lower than those in the normal control group (P<0.05). Treatment with CA markedly increased expression of GFAP in mice with DSS-induced colitis.

Effects of CA on p38 MAPK signal pathways

As shown in **Figure 3**, compared to the normal control group, treatment with DSS induced a marked increase in p-p38 MAPK expression. It was decreased with treatment of CA in mice with DSS-induced colitis. In addition, as shown in the **Table 4**, a significant increase in p-p38 MAPK expression, as assessed by ELISA, was observed in mice with DSS-induced colitis, compared to the control group (P<0.05). This increase in the amount of p-p38 MAPK in mice with DSS-induced colitis was decreased by treatment with low- or high-dose CA (P<0.05).

Discussion

To the best of our knowledge, this study is the first indicating that CA ameliorates DSS-induced colitis in mice, histologically and clinically, as assessed by histological injury scores and DAI. Beneficial effects of CA treatment may be linked, in part, to inhibition of p38 MAPK

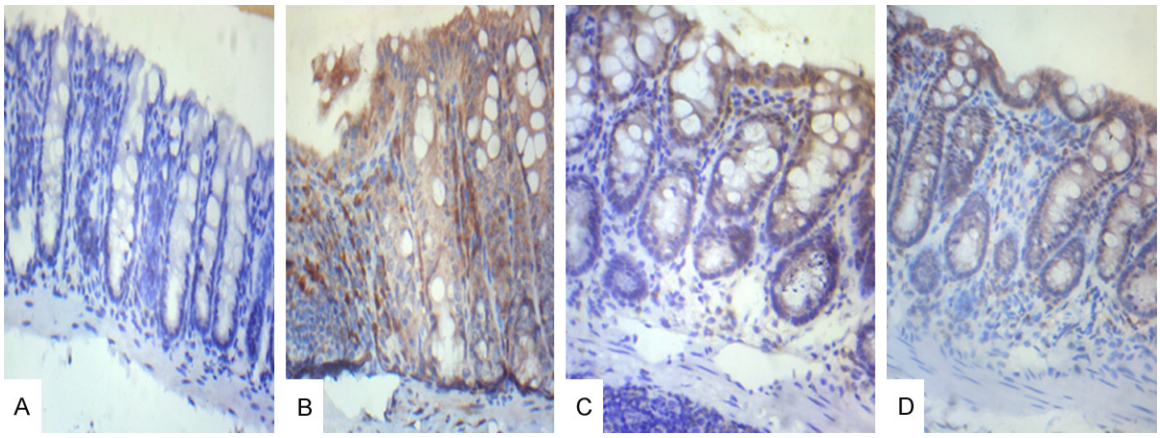

Figure 3. Effects of CA on p-p38 MAPK-positive cells. Immunohistochemical staining of p-p38 MAPK in colonic tissues (magnification ×400). A. Normal control group; B. DSS group; C. Low-dose CA group; D. High-dose CA group. Compared with normal controls, p-p38 MAPK protein expression was significantly increased in DSS group (P<0.05). After treatment with CA, the percentages of p-p38 MAPK-positive cells decreased significantly in mice with DSS-induced colitis compared with the DSS group (P<0.05). Most of the p-p38 MAPK-positive cells displayed cytoplasmic and nuclear staining.

Table 4. p-p38 MAPK expression in colonic tissues

Group	Number	p-p38 MAPK (pg/ml)
Normal control	8	0.32±0.15
DSS	8	7.36±0.58*
Low-dose CA	8	4.22±1.93
High-dose CA	8	3.65±0.84

Results are expressed as $\bar{x}$±s. *P<0.05 versus Normal control and CA group.

activity, regulation in enteric glia, and downregulating inflammatory mediators, such as IL-1β, TNF-α, and MPO activity. These findings suggest that CA may be a useful therapeutic approach for treatment of UC.

EGCs are critical regulators of gut homeostatic functions by modulating mucosal permeability, immune function, vascular tone, absorption, secretion, neural activity, and motility [1]. Indeed, selective depletion of EGCs leads to disruption of mucosal integrity, followed by severe mucosal inflammation [3, 15]. The present study shows that oral administration of 5% DSS induced predominant colitis and suppressed glial proliferation in colitis. Expression of GFAP and GDNF, correlating with the functional state of EGCs, were decreased in mice with DSS-induced colitis. Conversely, increased GFAP expression has been observed in inflammation [16]. Studies from the laboratory of von Boyen et al. have shown that GFAP and GDNF, as signs of activated EGCs, were increased in the inflamed mucosa of patients with UC [17]. In fact, opposite results in the present study may be due to severe inflammation and injury, such as high levels of TNF-α, IL-1β or nonimmune mechanisms [18]. In addition, previous experimental data have demonstrated that GDNF expression in colonic tissues increased in a time-dependent fashion after treatment with DSS, decreasing significantly at day 9 [13]. These data suggest that EGCs may play important roles in the pathogenesis of UC. Agents that selectively target EGCs may represent a novel approach in developing new therapeutic strategies for UC.

As described above, this study shows that colonic glia was destructed in DSS-induced colitis, but the mechanisms responsible for this impaired glial function are not clearly understood. It has been recognized that alterations in enteric glial function are often associated with mucosal inflammation [5, 6, 16, 17]. Indeed, inflammatory cytokines or bacterial compounds can increase GFAP or S100β expression [5, 16, 19]. In particular, IL-1β has been shown to dose-dependently decrease EGCs proliferation. IL-10 has concentration-dependent biphasic effects on EGCs proliferation, with low concentrations increasing and high concentrations reducing EGC proliferation [20]. On the other hand, EGCs can directly contribute to intestinal inflammatory response by produc-

ing inflammatory mediators and act as antigen-presenting cells. Additionally, EGCs are able to release high amounts of beneficial neurotrophic factors and neurotrophins in response to inflammatory stimuli [18]. Indeed, a previous study demonstrated that EGCs were able to regulate intestinal epithelial barrier integrity via their release of GDNF *in vivo* [13]. These results suggest that EGCs and inflammation are parts of complex regulatory networks that can activate or inhibit one another in multiple fashions. As described above, high levels of pro-inflammatory cytokines have been reported to disrupt EGCs [20]. Similarly, the present study demonstrated the development of severe inflammatory response in colonic tissues, characterized by upregulation of MPO activity, suggesting the recruitment of circulating leukocytes and the massive release of proinflammatory cytokines such as IL-1β and TNF-α. This may partly help to elucidate the mechanisms of impaired glial function in DSS-induced colitis. Thus, it was speculated that a reduction of excessive proinflammatory mediators would contribute to significant improvement of colonic glial function. Present data seem to support this postulation. Indeed, it was found that, accompanied by a marked decrease of these proinflammatory mediators after treatment with CA, DSS-induced defective glial function was ameliorated markedly.

Results showed that CA reduced inflammation and regulated EGCs in mouse models of dextran sulfate sodium-induced colitis. Similar results have been reported by Reddy et al. They observed CA exerted potent anti-inflammatory effects by inhibiting lipopolysaccharide-induced TNF-α and IL-6 mRNA expressions in RAW 264.7 macrophages [8]. Another report from Liu et al. also showed that CA inhibited lipopolysaccharide-induced expression of TNF-α and IL-1β in endothelial cells by suppressing MAPK activation [9]. Interestingly, present data showed treatment with CA markedly increased expression of GFAP and GDNF, which are involved in modulating the integrity of the bowels during inflammation [16]. Based on this data, it is reasonable to expect that CA protects EGCs from damage via preventing inflammation. In addition, the present study provides molecular mechanisms involved in CA-mediated enteric glial activation. For example, CA exert its regulation effects on EGCs via inhibition of p38 MAPK pathways. Results showed that CA inhibited p-p38 MAPK, a marker of p38 MAPK pathway activation. Similar results have been reported by Reddy and Liu et al. [8, 9]. It has been established that expression of S100β protein and p38 pathway signaling molecules are upregulated in EGCs deriving from DSS-treated mice and UC patients [21]. Therefore, it is possible that CA mediates glia-protective effects, at least in part, via inhibition of p38 MAPK pathways.

In summary, CA treatment can effectively reduce inflammation and improve glial function in mouse models of dextran sulfate sodium-induced colitis. However, this study did not evaluate S100β secretion, glial cells apoptosis, and autophagy. Another limitation of this study was that it did not compare CA with other positive control drugs, such as mesalamine or glucocorticosteroids. Considering less toxicity, easy availability, and multi-target integrated regulation of CA, further studies are required to better define the CA anti-inflammatory effects and involvement of EGCs in UC onset, progression, and carcinogenesis.

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Disclosure of conflict of interest

None.

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